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Formation of a Cross-Linking Complex of Dinitrogenase Reductase-Activating Glycohydrolase (DRAG) with Membrane Proteins from *Rhodospirillum rubrum* Chromatophores

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Abstract—Association of dinitrogenase reductase-activating glycohydrolase (DRAG) with membrane proteins of chromatophores has been investigated. The formation of a multicomponent complex between DRAG and membrane proteins was demonstrated in the presence of glutaraldehyde and EDC/NHS (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride/hydroxy-2,5-dioxopyrrolidine-3-sulfonic acid sodium salt). Complex formation was observed both in native chromatophore membrane and in chromatophores treated with 0.5 M NaCl in the presence of homogeneous DRAG and glutaraldehyde in cross-reaction. The molecular weight of the complex was around 200 kD, which is consistent with the association of DRAG with three or more chromatophore membrane proteins. A specific complex with molecular weight of about 75 kD was formed only in the presence of EDC/NHS in the cross-linking reaction. It was demonstrated that ammonium transport protein and P11 protein are possible candidates for association with DRAG in chromatophore membranes.

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Nitrogen fixation is catalyzed by the enzyme nitrogenase (EC 1.18.6.1) in all diazotrophs studied [1]. Nitrogenase consists of dinitrogenase reductase (Fe protein) and dinitrogenase (MoFe protein) which are both required for the enzyme activity. Dinitrogenase reductase transfers electrons from an electron donor to the dinitrogenase, which then can catalyze the reduction of nitrogen to ammonium. The nitrogen fixation is an energy-demanding process, and it is strictly regulated at both the transcriptional and posttranslational levels.

Transcriptional regulation of *nif* (nitrogenase fixation) genes has been observed in all diazotrophs, and it is best characterized in *Klebsiella pneumoniae*, a free-living nitrogen fixing bacterium [2]. Besides the transcriptional regulation, a posttranslational mechanism for regulation of nitrogenase activity was demonstrated in several types of diazotrophs in response to various environmental conditions [3, 4]. This phenomenon has been termed a "switch-off" effect. The switch-off occurs when fixed nitrogen is available as ammonium or glutamine, or when insufficient light energy is available to perform the ATP-demanding reduction of N_2 [5]. Posttranslational regulation of nitrogen fixation has been well characterized in *Rhodospirillum rubrum*, *Azospirillum brasilense*, *Azospirillum lipoferum*, and *Rhodobacter capsulatus*, where it involves reversible mono-ADP ribosylation of dinitrogenase reductase [3-12] in response to ammonium addition or darkness or anaerobiosis.

Dinitrogenase reductase-ADP-ribosyl transferase (EC 2.4.2.37, referred to here as DRAT, the gene product of *draT*) transfers ADP-ribose from NAD^+ to the Arg101 residue of one subunit of the dinitrogenase reductase homodimer, resulting in inactivation of that enzyme [4, 13].

Dinitrogenase reductase-activating glycohydrolase (EC 3.2.2.24, referred to here as DRAG, the gene prod-

Abbreviations: DRAG) dinitrogenase reductase-activating glycohydrolase; DRAT) dinitrogenase reductase-ADP-ribosyltransferase; EDC) N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; IPTG) isopropyl β-D-thiogalactopyranoside; LB) Luria–Bertani medium; PVDF) Tropifluor™ polyvinylidene difluoride membrane; sulfo-NHS) hydroxy-2,5-dioxopyrrolidine-3-sulfonic acid sodium salt.

uct of *draG*) removes the ADP-ribose group attached to dinitrogenase reductase, thus restoring nitrogenase activity [14-16]. *Rhodospirillum rubrum* and *Rba. capsulatus* strains mutated in *draT* and *draG* have been reported to be unable to regulate *in vivo* nitrogenase activity in response to NH_4^+ and darkness [17].

However, the relationships between the switch-off effect and the *draTG* system (genes coding DRAT and DRAG enzymes) are more complicated. In some photosynthetic bacteria, switch-off occurs while the *draTG* system is absent [18, 19]. For example, it was shown that photosynthetic *Rba. sphaeroides* appears to have no *draTG* genes [19, 20]. However, transfer of *draTG* from *Rba. capsulatus* conferred on *Rba. sphaeroides* the ability to reversibly modify the nitrogenase Fe-protein in response to ammonium or darkness. At the same time, introduction of *draTG* had no significant effect on kinetics of the switch-off/on process [19]. These data demonstrated that an ADP-ribosylation-independent nitrogenase regulatory pathway exists. In addition, it has been shown that even in bacteria with the *draTG* system, e.g. *Rba. capsulatus*, the switch-off effect was not connected with *draTG*, but had a similar non-ADP-ribosylation-dependent regulatory mechanism [9, 20, 21]. Furthermore, strong evidence has been provided that the deletion of the *draTG* region did not alter the switch-off effect in *Rba. capsulatus* [22]. These results indicate that two different regulatory systems can coexist with the same apparent function.

In addition, several studies have shown that the purple bacterium *Rba. capsulatus* manifests a different, presumably independent, control mechanism of posttranslational regulation [23, 24]. It has been demonstrated that highly nitrogen-limited cultures of both wild-type strain and *draT/draG* mutant carried out the nitrogenase switch-off, while moderately nitrogen-limited cultures showed instead an ADP-ribosylation-independent "magnitude" response. In this case, the nitrogenase magnitude response was accompanied by the decrease of *in vivo* nitrogenase activity, where the quantity of added NH_4^+ affects the magnitude of the inhibition [23].

Recently it has also been shown that response to ammonium depends on the growth history of *Rba. capsulatus* [24]. It was clearly demonstrated that the presence of ADP-ribosylated Fe-protein is correlated with different nitrogen sources, e.g. ADP-ribosylated Fe-protein was found in both exponential (N_2, glutamate) and stationary (NH_4^+-limited) growth phases. It was concluded that the modification state of Fe-protein was directly proportional to the level of intracellular glutamine, and therefore it was presumably a function of the cellular nitrogen status.

In summary, it appears there are at least two different mechanisms of the switch-off/switch-on effect. One is uniquely mediated by the DRAT/DRAG system and covalent modification/demodification of the Fe-protein, and the second is based on metabolic modulation of nitrogenase activity in response to NH_4^+ addition without covalent modification.

Furthermore, the interaction between DRAT and DRAG enzymes also depends upon environmental stimuli [16]. DRAT is inactive and DRAG is active under nitrogen-fixing conditions, and dinitrogenase reductase is in its active state. When the negative stimuli, such as exogenous ammonium ions or energy depletion, are added, DRAT becomes active rapidly, and DRAG is inactivated. This results in the loss of nitrogenase activity and the modification of dinitrogenase reductase. However, DRAT is only transiently active, and it becomes inactive again even in the presence of a negative stimulus. At the same time, DRAG stays inactive as long as the effector is present, until ammonium is metabolized, or cells are subjected to light. After removal of a negative stimulus, DRAG becomes active again, and dinitrogenase reductase is reactivated by cleavage of the ADP-ribose group. The switch-off effect is never complete—usually some 10% of the original activity remains, and fully active DRAT and totally inactive DRAG are not present at the same time. During the recovery of nitrogenase activity DRAG is activated and remains active, when switch-off effectors are removed [25]. The mechanism of regulation of DRAT and DRAG activities is still unknown.

A full understanding of the metabolic regulation of nitrogenase requires the identification of the intracellular signals from a switch-off effector to DRAT and DRAG. The membrane association of DRAG can be the key event in the switch-off effect. DRAG was originally isolated as a nitrogenase-activating enzymatic activity found in the pellet fraction of *Rsp. rubrum* [15, 26]. The intracytoplasmic membranes, termed chromatophores, are packed with the photosynthetic machinery of the cell and would presumably be the site of interaction for DRAG. Earlier it was shown that DRAG is a membrane-associated protein, and it can be removed from chromatophore membranes by treatment with 0.5 M sodium chloride or GDP [27]. It was proposed that regulation of DRAG could be mediated by the association/dissociation to the chromatophore membrane. DRAG would be inactive when associated to the membrane and active when dissociated. If this association is physiologically important, it could also be specific, and DRAG could be associated to a protein or protein complex in the membrane. The formation of high molecular mass protein complex has been shown earlier [28]. This complex contained DRAG after treatment of chromatophores with glutaraldehyde cross-linker. However, the interacting membrane proteins have not been identified.

Moreover, it was found that purified DRAG binds reversibly to purified, native membranes of *Rsp. rubrum* lacking DRAG [29]. The existence of a protein requirement for DRAG binding was suggested, because DRAG did not bind significantly to phospholipid vesicles. DRAG

was bound to salt-washed membranes with an affinity equal to that of native membranes. These results indicated that DRAG directly binds membrane components. However, the complex of DRAG with the membrane is still unknown and not directly identified.

Therefore, the goal of the present work was to establish an association of DRAG with chromatophore membrane proteins and identify them. Cross-linking studies were carried out when we wanted "to trap" a membrane protein candidate for DRAG binding.

MATERIALS AND METHODS

The following chemicals were used in this study: $Na_2S_2O_4$, hydroxy-2,5-dioxopyrrolidine-3-sulfonic acid sodium salt (sulfo-NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), and isopropyl β-D-thiogalactopyranoside (IPTG) from Sigma (USA). Reagents for SDS-PAGE and Western blot were from Amersham (USA). Novex Colloidal Coomassie G-250 kit was from Invitrogen (USA). Antibodies for P11 and AmtB were kindly provided by M. Johansson and M. Merick (JIC Norwich, UK), respectively. His-DRAG construct was prepared by A. Noren (Stockholm University, Sweden). Other reagents were of analytical grade and used without further purification.

Growth conditions. *Rhodospirillum rubrum*, strain S1, was grown photoheterotrophically to A_{600} = 1.5-2.0, under an atmosphere of N_2/CO_2 (95 : 5) as described earlier [30].

Protein purification. Nitrogenase (MoFe protein) was purified from strain S1, wild type *Rsp. rubrum*. Cells were harvested, resuspended in 0.1 M Tris-HCl, pH 7.6, and broken in a Ribi Cell fractionator as described previously [31]. ADP-ribosylated Fe protein (inactive) was purified from strain S1, *Rsp. rubrum* cells, as described in [32]. Samples were kept in liquid nitrogen before use.

DRAG assay. The activity of His-DRAG and DRAG in chromatophores was measured indirectly as the activation of inactive nitrogenase assayed as acetylene reduction [33, 34]. Nitrogenase activity is expressed as μmoles of ethylene formed per ml of cell culture per hour, normalized to a cell culture with A_{600} of 1.0. DRAG activity was determined using modified nitrogenase-coupled DRAG assay, in which the removal of ADP-ribose was differentiated from the acetylene reduction. The standard demodification step was conducted in an anaerobic, stoppered vial containing 5 mM ATP, 25 mM phosphocreatine, 50 μg of creatine phosphokinase, 25 mM $MgCl_2$, 0.5 mM $MnCl_2$, 10 mM $Na_2S_2O_4$, 20-40 μg of ADP-ribosylated Fe protein, 350 ng of DRAG, and 50 mM Tris-HCl, pH 7.8, in total volume of 500 μl under nitrogen headspace. The acetylene reduction step was initiated by the addition of any components excluded from the demodification step, along with 75 μg of MoFe protein and 10% acetylene in the headspace. The headspace was analyzed for ethylene content by gas chromatography.

Purification of His-DRAG. His-DRAG was purified according to the general protocol kindly provided by A. Noren (Stockholm University, Sweden). We used the plasmid pWPF 109 in DH5α carrying a 5.2 kb fragment of *Rsp. rubrum draT/G* ORF and nif HD′ region in pUC 19 vector as a source for *draG*. Primers were constructed for *draG* with *Nde* I site at the 5′ end. After this step, the PCR product was cloned into pET-28a(+) vector containing a histidine tag at the N-terminal of DRAG. The vector *draG* was overexpressed in pET-28a(+) vector using T7 promoter and lacI. The expression of the "target" gene was analyzed by SDS-PAGE followed by Coomassie Blue staining. draG-pET-28a(+) was transformed into BL21(DE3)pLysS. Expression of His-DRAG was induced by IPTG. LB medium containing the kanamycin (final concentration 25 μg/ml) and chloramphenicol (final concentration 35 μg/ml) was inoculated with single colonies. The growth media has been incubated with shaking at 37°C until the A_{600} = 0.6 (about 3-4 h). The final concentration of IPTG was 0.4 mM. Vials were placed on ice for 5 min, and then the cells were harvested by centrifugation at 5000*g* for 5 min at 4°C. Finally, cells were resuspended in 0.1 M Tris-HCl, pH 7.8, at 25°C and centrifuged as above. The cell pellet was collected. It was resuspended again in ice-cold binding buffer (40 mM imidazole, 4 M NaCl, 160 mM Tris-HCl, pH 7.9). The cell suspension was supplemented with $MgCl_2$ (10 mM), DNase (20 μg/ml), and protease inhibitors and incubated at room temperature for 20 min. The cells were sonicated with 5-20-sec pulses until the solution became nonviscous. The lysate was centrifuged at 40,000*g* for 20 min to remove cell debris. The clear lysate was applied to a Ni^{2+}-affinity column (10 × 1.5 cm) equilibrated with 10 volumes of the binding buffer. The flow rate was about 10 column volumes per hour. The column was washed with six volumes of 480 mM imidazole, 4 M NaCl, 160 mM Tris-HCl, pH 7.9 (wash buffer). The His-DRAG was eluted with six volumes of 4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9 (elution buffer). DRAG was tested for purity by SDS-PAGE and stained with Coomassie Blue using a Novex Colloidal Coomassie G-250 kit (Invitrogen). Fractions containing His-DRAG were combined and used for further experiments.

Preparation of chromatophores. *Rhodospirillum rubrum*, strain S1, was grown diazotrophically as described previously [30]. Cells were harvested, resuspended in anaerobic 0.1 M Tris-HCl, pH 7.6 (buffer A), and disrupted in a Ribi Cell fractionator. Two different preparations of chromatophores were used.

1. Preparation of native chromatophores with DRAG: after removal of undisrupted cells, chromatophores were pelleted by centrifugation at 45,000*g* for 90 min. The chromatophores were washed twice by resus-

pension in anaerobic buffer A and centrifuged at 45,000*g* for 90 min.

2. Preparation of chromatophores without DRAG: chromatophore pellet was washed twice with buffer A containing 0.5 M NaCl to dissociate DRAG from the chromatophores. The chromatophores were kept in liquid nitrogen before use.

Protein concentration. Protein concentration was determined according to [35].

Cross linking of DRAG in native chromatophore membranes. Native chromatophore membranes containing DRAG were resuspended in 14 mM Hepes, pH 7.6, and incubated for 2 h at 4°C, and then 8 mM sulfo-NHS and 32 mM EDC were added. Samples were incubated at room temperature for 22 h. The EDC was then quenched with 0.5 M Tris solution. The chromatophore membranes were solubilized by addition of SDS buffer containing 130 mM Tris-HCl, pH 6.8, 4.2% (w/v) SDS, 20% (v/v) glycerol, 0.003% (w/v) bromophenol blue, and 10% (v/v) 2-mercaptoethanol (freshly added). After incubation at 90°C for 15 min, 10 µl (for DRAG immunoblots) or 15 µl (for Coomassie or silver staining) of each reaction sample was loaded onto 9% SDS-PAGE. Separated proteins were either Coomassie stained [36] or electrophoretically transferred onto PVDF (Tropifluor™ polyvinylidene difluoride) membrane, which was incubated with polyclonal antibodies against DRAG (1 : 10,000, overnight) or against AmtB (1 : 500, overnight) and P11 (1 : 500, overnight) proteins. The blot was then incubated with anti-rabbit immunoglobulins, horseradish peroxidase-linked whole antibody (1 : 5000, 30 min) and used for enhanced chemiluminescence detection of proteins according to the manufacturer's instructions. Cross-linking experiments were repeated 3-5 times.

Cross-linking of His-DRAG in chromatophore membranes treated with 0.5 M sodium chloride. Before cross-linking chromatophores were resuspended in 14 mM Hepes, pH 7.6. His-DRAG was pre-incubated with chromatophore membranes on ice for 2 h. Then glutaraldehyde (10 mM final concentration) was added in 14 mM Hepes, pH 7.6. Samples were kept at 4°C. The reaction was quenched with 0.5 M Tris after 24 h. The incubation mixture was analyzed for DRAG activity assays, as well as by SDS-PAGE and Western blotting as described above. Cross-linking experiments were repeated 3-5 times.

RESULTS AND DISCUSSION

We studied the association of active DRAG with chromatophore membrane proteins. To obtain highly purified DRAG for protein–protein interaction analysis, and more important, to obtain DRAG with full biological activity, one-step purification using metal-column affinity chromatography was developed. His-tagged DRAG was obtained by cloning and overexpression in *E. coli* and was purified by nickel affinity chromatography (table). The purification procedure including one step of Ni^{2+}-affinity chromatography did differ significantly from the protocols described in the literature.

The purity of His-DRAG protein was demonstrated by SDS-PAGE (Fig. 1). His-DRAG was purified to homogeneity (lanes *2* and *3*), judged by SDS-PAGE, and molecular weight was determined to be near 32 kD as expected for DRAG. The localization of DRAG was further confirmed by Western blotting using anti-DRAG monoclonal antibodies (Fig. 1, lane *4*). The biological activity of His-DRAG has been shown as the activation of inactive nitrogenase (Fe protein) assayed as acetylene reduction [33, 34]. The activity was 655 µmol of C_2H_4/ml per h.

To identify the proteins interacting with His-DRAG in chromatophore membranes, we carried out the following experiments. It has already been shown that an addition of 0.5 M sodium chloride completely removed DRAG from chromatophore membranes [27]. Based on this we treated chromatophore membranes with 0.5 M sodium chloride to obtain DRAG-free chromatophore membranes. Then His-tagged DRAG was added to DRAG-free membrane and treated with glutaraldehyde, the cross-linking reagent. The reaction mixture was then analyzed by Western blotting. The result of cross-linking experiment is shown in Fig. 2. The DRAG-free chromatophore membranes did not show any DRAG activity (control, Fig. 2, lane *1*). However, addition of His-DRAG to these membranes and incubation in the presence of glutaraldehyde result in formation of cross-linking complex (Fig. 2, lane *2*). Incubation of PVDF membrane with anti-DRAG monoclonal antibodies identified the formation of a multicomponent complex with molecular weight of about 200 kD (Fig. 2, lane *2*). Without glutaraldehyde treatment, no complex was observed on

Purification of His-DRAG from *E. coli*

Step of purification	Yield, mg	Specific activity, µmol C_2H_4/h per mg protein	Total activity, µmol C_2H_4/h	Purification, fold
Crude extract	190	12.6	2393.1	1
Eluate from Ni^{2+}-NTA column	3.3	655.3	2129.8	52

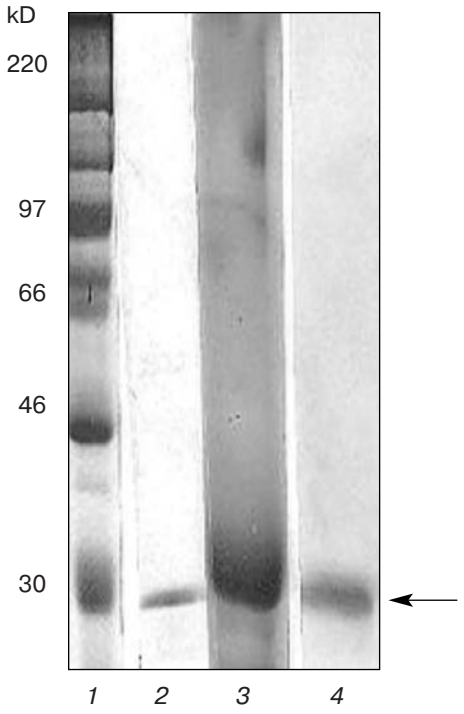

Fig. 1. Purification of His-DRAG from *E. coli*. Coomassie Blue-stained 10% SDS gel: *1*) marker; *2*) 4 M imidazole eluate containing pure His-DRAG. *3*) Silver-stained 10% SDS gel; *4*) Western blot of His-DRAG. Arrow shows the position of His-DRAG.

To perform *in vivo* binding of DRAG to chromatophores membrane proteins, we used intact system, i.e. native chromatophores, already containing DRAG. When native chromatophores were added to EDC/NHS mixture, formation of protein complexes was observed, which migrated at about 75 and 200 kD. Both bands cross-reacted with anti-DRAG antibodies (Fig. 3, lane *1*). However, when EDC/NHS was excluded from the reaction mixture no cross-linking complex was observed on DRAG immunoblot (Fig. 3, lane *2*). This control provides evidence that cross-linking complexes with molecular weight of 75 and 200 kD were the result of covalent linkage between the DRAG and chromatophore membrane proteins. Our data demonstrated that large 200 kD complex was always produced in cross-linking reaction, and its formation did not depend upon the specificity of the cross-linking reagent. In contrast, smaller of 75 kD complex is an EDC-dependent cross-linking product. Its formation was demonstrated only in the presence of the highly specific cross-linking reagents. It is likely this complex is very specific and unique, and it provides a possibility for identification of DRAG-interacting proteins. In summary, the results of cross-linking studies have shown the association of DRAG with two or more proteins from chromatophore membranes.

The next step of this study was identification of these proteins, possible candidates in protein–protein interactions. Previously it was shown that P11 protein, its DRAG immunoblot (Fig. 2, lane *3*). In control experiments, the individual His-DRAG did not form dimers or trimers during incubation with glutaraldehyde, and no aggregation of DRAG was observed (Fig. 2, lane *4*). The cross-linked complex did not show any enzyme activity.

These data have demonstrated that DRAG could produce a multi-protein complex with chromatophore membranes. The formation of high molecular weight protein complex (about 200 kD) was shown earlier [28]. The complex contained DRAG and formed after treatment of chromatophores with glutaraldehyde. So, in the presence of glutaraldehyde DRAG can interact and associate with three or even four membrane proteins, since glutaraldehyde is not a specific cross-linking agent, and more than one protein can be involved in the formation of a cross-linking complex. However, in such a multi-protein mixture it is difficult to identify particular proteins that might be important for the regulation of DRAG activity.

Therefore, in further experiments we used more specific cross-linkers, such as EDC and sulfo-NHS. In the presence of sulfo-NHS, EDC modifies carboxyl group of protein, which then react with ε-amino group of lysine residue in an interacting protein, forming a covalent peptide bond between interacting proteins. EDC is a zero-length cross-linker; since no spacer group is utilized, the proteins being bound via existing functional groups.

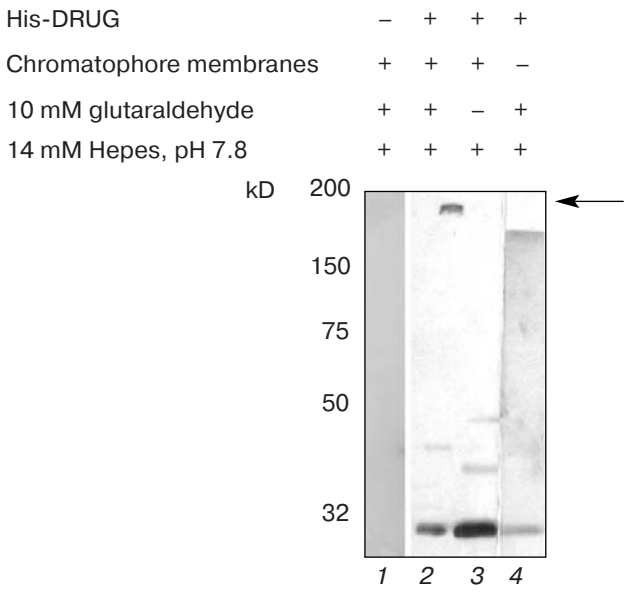

Fig. 2. Western blot (9% SDS gel) showing the cross-linking reaction of DRAG with 0.5 M NaCl-treated chromatophore membranes (lane *2*). The complete cross-linking reaction was performed as described in "Materials and Methods". Lanes *1*, *3*, and *4* contain control cross-linking reaction mixtures with one component excluded (–) as indicated above the lanes. The arrow indicates the position of the identified protein species.

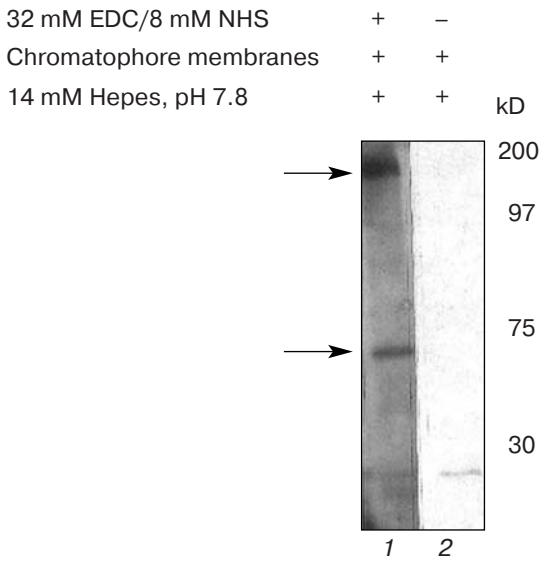

Fig. 3. Western blot of cross-linked DRAG complex formed with membrane proteins from native chromatophores (in 9% SDS gel). Lanes: *1*) complete cross-linking mixture; *2*) reaction mixture minus EDC/NHS. Arrows indicate the positions of the identified protein species.

homologs, and ammonium transport protein (AmtB) affected posttranslational regulation of nitrogenase activity in *Rsp. rubrum*, *A. brasilence*, and *Rba. capsulatus* [16, 37-40].

P11 proteins are among the most highly conserved and widely distributed signal transducers occurring in bacteria [41], *Archaea* [42, 43], as well as in the chloroplasts of eukaryotic algae and plants [44]. The AmtB proteins constitute a family of ammonia channel proteins found in all domains of life. They have been found in eubacteria, archaebacteria, fungi, plants, and animals including humans [45]. We assumed that these two proteins, AmtB and P11, can participate in posttranslational regulation of nitrogenase activity and interact with the DRAG/DRAT system.

It was shown earlier that a *Rsp. rubrum* strain with an alteration in *glnB* (gene coding P11-Y51F protein, where the tyrosine that serves as the site of uridylylation was altered) had low nitrogenase activity, and small effects on the regulation of DRAT activity were observed [46]. In *Rsp. rubrum* and *A. brasilense*, *ntrBC* mutations have no effect on *nif* expression, whereas in *K. pneumoniae* NtrBC proteins are required for *nif* expression [38-40]. It has been shown that *ntrBC* mutations in *A. brasilense* did alter the regulation and cause slower inactivation of DRAG activity by ammonium ions [11]. This indicates that some parts of the *ntr* system are involved in DRAG regulation. In *Klebsiella pneumoniae*, P11 also affects the regulation of DRAT and DRAG activity, but less dramatically than GlnK does. The absence of GlnK alters DRAG, but not DRAT, and this effect is independent on the level of DRAG [16]. In *Rsp. rubrum*, reversible membrane association of DRAG is mediated by AmtB1 and GlnJ proteins [47]. Besides this, AmtB has been shown to be a sensitive test of the ammonium ion concentrations in *E. coli* [48].

Because of their key roles in the *ntr* system in response to an ammonium signal, P11 and AmtB are reasonable candidates for the interaction with DRAG and might actually affect DRAT/DRAG regulation. P11 and AmtB were therefore examined for their participation in interaction and formation the cross-linking complexes with DRAG. We added P11 and AmtB antibodies to the cross-linking complex of DRAG in native chromatophores. The results of immunoblot showed the cross-linking complex with molecular weight of 75 kD had strong reaction with AmtB antibodies and less signal with P11 antibodies (Fig. 4, lanes *2-4*). The molecular weight of the complex (about 75 kD) corresponds to the sum of molecular weights of DRAG (32 kD) [15], AmtB protein monomer (30 kD) [49], and P11 protein monomer (14 kD) [50]. We would like to note that this protein–protein interaction complex has been identified in the presence of specific cross-linking agents (EDC/NHS) in native chromatophore membranes. These data show that AmtB and P11 proteins can bind with DRAG in the cross-linking reaction. Further studies are needed to identify proteins in large cross-linking complex with molecular weight of 200 kD.

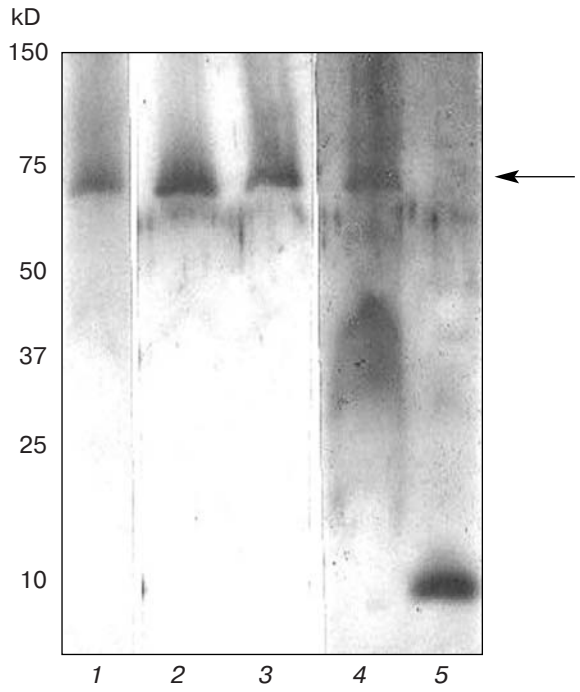

Fig. 4. Immunoblot of 9% SDS gel showing the reaction of cross-linking complex DRAG in native chromatophore membranes with DRAG antibodies (*1*), AmtB antibodies (*2*, *3*), P11 antibodies (*4*); *5*) pure P11. The arrow indicates the position of the cross-linking complex.

In summary, our research has demonstrated the association of DRAG with chromatophore membrane proteins and the formation of cross-linking complex. Our data indicate AmtB and P11 are the most perspective candidates for the interaction with DRAG. Further investigation will focus on mass spectrometry analysis of this cross-linking complex to characterize these proteins and confirm our data. These studies are needed to understand the molecular details of the mechanism of DRAG association/dissociation and its regulation.

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